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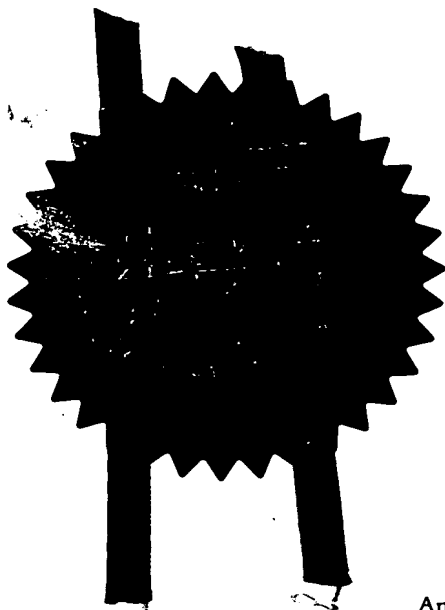
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Patents ADP number (if you know it)

7223522001

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4. Title of the invention

DUAL-VIRUS VECTORS

5. Name of your agent (if you have one)

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## DUAL-VIRUS VECTORS

This invention relates to improved viral vector systems for delivery of genetic material to target cells. The invention also relates to the target cells containing the delivered genetic material. The invention further  
5 relates to methods of treatment using the vector systems and/or the target cells.

The delivery of genes with therapeutic or prophylactic activity to patients or those at risk of contracting a particular disease or viral  
10 infection has great potential for the prevention or cure of a wide variety of illnesses. However the currently available genetic vectors for delivery of genes into the individual to be treated have significant limitations (reviewed by Roth and Cristiano 1997 J. Nat. Cancer Inst. 89: 21-39).

The majority of gene-transfer protocols for cancer gene  
15 therapy employ defective retroviral vectors (reviewed by Roth and Cristiano 1997 J. Nat. Cancer Inst. 89: 21-39). These have significant advantages for some applications compared with other viral and non-viral delivery systems: they can achieve stable integration and expression of the  
delivered genes in the target cell and do not generally trigger a significant  
20 immune response against the vector. The retroviral vectors are rendered defective by the deletion of one or more genes which are essential to complete the life-cycle of the virus. The defective genome is propagated in a helper cell line cultured *in vitro*. The helper cell line is engineered to contain viral genes capable of complementing the defect in the vector  
25 genome. Thus vector particles produced on introduction of DNA capable of expression of defective vector genome into the helper cell line are capable of a single round of transduction of a target cell population but cannot replicate in the target cell and spread to other cells. This is an important safety feature which limits the spread of virus to non-target  
30 tissues, other organs and the environment.

The practical uses of retroviral vectors are limited largely by the titres of transducing particles which can be attained in *in vitro* culture (typically not more than  $10^8$  particles/ml) and the sensitivity of many enveloped viruses to traditional biochemical and physicochemical techniques for concentrating and purifying viruses.

Other types of viruses have also been used to develop vectors for gene delivery including Adenoviruses, Adeno-associated virus (AAV), Herpes viruses, Pox viruses. Defective viral vectors which are capable of only a single round of transduction of target cells have been constructed from members of each of these families of virus. Since the target cells lack at least one essential function for production of infectious viral particles, further spread of the viral vector is prevented in such cases. Vectors based on Adenovirus and Pox virus can be produced at high titre and are relatively stable during purification and storage compared with enveloped viruses but tend to be immunogenic. This may be an advantage for certain prophylactic vaccine indications but limits the sustained expression of therapeutic genes needed for many applications. The titres obtained with such viral vectors are also not high enough to achieve an effective therapeutic index in many cases since *in vivo* delivery leads to uptake of the vector and expression of the delivered genes in only a proportion of target cells.

A single round of viral transduction is a feature of defective retroviral and defective non-retroviral vectors which is shared with non-viral gene delivery systems such as liposomes, immunoliposomes and non-viral systems containing compacted DNA.

The use of all of these viral and non-viral delivery systems is thus limited by the efficiency of gene transfer to target cells *in vivo* and in the case of cancer treatment, is limited particularly by inadequate penetration of gene delivery vehicles throughout the tumour tissue.

Another limitation of currently available viral vectors relates to safety of the viral vectors *in vivo*. In many cases, just one or two recombination events among the nucleic acids encoding the viral vectors would be sufficient to produce an infectious virus particle capable of replication. This poses too great a risk for *in vivo* use.

It is an object of the present invention to provide a defective genetic vector which is capable of limited spread *in vivo* via viral transduction.

It is a further object of the present invention to provide defective genetic vectors which incorporate safety features for *in vivo* use.

In one aspect, the invention provides a hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell.

The genetic vector of the invention is thus a hybrid viral vector system for gene delivery which is capable of generation of defective infectious particles from within a target cell. Thus a genetic vector of the invention consists of a primary vector manufactured *in vitro* which encodes the genes necessary to produce a secondary vector *in vivo*. In use, the secondary vector carries one or more selected genes for insertion into the secondary target cell. The selected genes may be one or more marker genes and/or therapeutic genes. Marker genes encode selectable and/or detectable proteins.

In another aspect the invention provides target cells infected by the primary viral vector or vectors and capable of producing infectious secondary viral vector particles.

In a further aspect the invention provides a method of treatment of a human or non-human mammal, which method comprises

administering a hybrid viral vector system or target cells infected by the primary viral vector or vectors, as described herein.

The primary viral vector or vectors may be a variety of different viral vectors, such as retroviral, adenoviral, herpes virus or pox virus vectors, or in the case of multiple primary viral vectors, they may be a mixture of vectors of different viral origin. In whichever case, the primary viral vectors are preferably defective in that they are incapable of independent replication. Thus, they are capable of entering a target cell and delivering the secondary vector sequences, but not of replicating so as to go on to infect further target cells.

In the case where the hybrid viral vector system comprises more than one primary vector to encode the secondary vector, both or all three primary vectors will be used to infect a primary target cell population, usually simultaneously. Preferably, there is a single primary viral vector which encodes all components of the secondary viral vector.

The preferred single or multiple primary viral vectors are adenoviral vectors. Adenovirus vectors have significant advantages over other viral vectors in terms of the titres which can be obtained from *in vitro* cultures. The adenoviral particles are also comparatively stable compared with those of enveloped viruses and are therefore more readily purified and stored. However, current adenoviral vectors suffer from major limitations for *in vivo* therapeutic use since gene expression from defective adenoviral vectors is only transient. Because the vector genome does not replicate, target cell proliferation leads to dilution of the vector. Also cells expressing adenoviral proteins, even at a low level, are destroyed by an immunological response raised against the adenoviral proteins.

The secondary viral vector is preferably a retroviral vector. The secondary vector is produced by expression of essential genes for assembly and packaging of a defective viral vector particle, within the primary target cells. It is defective in that it is incapable of independent

replication. Thus, once the secondary retroviral vector has transduced a secondary target cell, it is incapable of spreading by replication to any further target cells.

The secondary vector may be produced from expression of essential genes for retroviral vector production encoded in the DNA of the primary vector. Such genes may include a gag-pol gene from a retrovirus, an envelope gene from an enveloped virus and a defective retroviral genome containing one or more therapeutic genes. The defective retroviral genome contains in general terms sequences to enable reverse transcription, at least part of a 5' long terminal repeat (LTR), at least part of a 3'LTR and a packaging signal.

Importantly, the secondary vector is also safe for *in vivo* use in that incorporated into it are one or more safety features which eliminate the possibility of recombination to produce an infectious virus capable of independent replication.

To ensure that it is replication defective the secondary vector may be encoded by a plurality of transcription units, which may be located in a single or in two or more adenoviral or other primary vectors. Thus, there may be a transcription unit encoding the secondary vector genome, a transcription unit encoding gag-pol and a transcription unit encoding env. Alternatively, two or more of these may be combined. For example, nucleic acid sequences encoding gag-pol and env, or env and the genome, may be combined in a single transcription unit. Ways of achieving this are known in the art.

Transcription units as described herein are regions of nucleic acid containing coding sequences and the signals for achieving expression of those coding sequences independently of any other coding sequences. Thus, each transcription unit generally comprises at least a promoter, an enhancer and a polyadenylation signal. The promoter and enhancer of the transcription units encoding the secondary vector are preferably strongly

active, or capable of being strongly induced, in the primary target cells under conditions for production of the secondary viral vector. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity. Examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a MUC1 gene, a CEA gene or a 5T4 antigen gene. Examples of temporally restricted promoters/enhancers are those which are responsive to ischaemia and/or hypoxia, such as hypoxia response elements or the promoter/enhancer of a grp78 or a grp94 gene. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

Hypoxia or ischaemia regulatable expression of secondary vector components may be particularly useful under certain circumstances. Hypoxia is a powerful regulator of gene expression in a wide range of different cell types and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1; Wang & Semenza (1993). *Proc. Natl. Acad. Sci USA* 90:430), which bind to cognate DNA recognition sites, the hypoxia-responsive elements (HREs) on various gene promoters. Dachs et al. (1997). *Nature Med.* 5: 515.) have used a multimeric form of the HRE from the mouse phosphoglycerate kinase-1 (PGK-1) gene (Firth et al. (1994). *Proc. Natl. Acad. Sci USA* 91:6496-6500) to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (Dachs et al. *ibid*). Alternatively, the fact that marked glucose deprivation is also present in ischaemic areas of tumours can be used to activate heterologous gene expression specifically in tumours. A truncated 632 base pair sequence of the grp 78 gene promoter, known to be activated specifically by glucose deprivation, has also been shown to be

capable of driving high level expression of a reporter gene in murine tumours *in vivo* (Gazit G, et al. (1995). Cancer Res. 55:1660).

Safety features which may be incorporated into the hybrid viral vector system are described below. One or more such features may  
5 be present.

Firstly, sequence homology between the sequences encoding the components of the secondary vector may be avoided by deletion of regions of homology. Regions of homology allow genetic recombination to occur. In a particular embodiment, three transcription  
10 units are used to construct a secondary retroviral vector. Transcription unit 1 contains a retroviral gag-pol gene under the control of a non-retroviral promoter and enhancer. Transcription unit 2 contains a retroviral env gene under the control of a non-retroviral promoter and enhancer. Transcription unit 3 comprises a defective retroviral genome under the control of a non-  
15 retroviral promoter and enhancer. In the native retroviral genome, the packaging signal is located such that part of the gag sequence is required for proper functioning. Normally when retroviral vector systems are constructed therefore, the packaging signal, including part of the gag gene,  
remains in the vector genome. In the present case however, the defective  
20 retroviral genome contains a minimal packaging signal which does not contain sequences homologous to gag sequences in transcription unit 1. Also, in retroviruses, for example Moloney Murine Leukaemia virus (MMLV), there is a small region of overlap between the 3' end of the pol coding sequence and the 5' end of env. The corresponding region of  
25 homology between transcription units 1 and 2 may be removed by altering the sequence of either the 3' end of the pol coding sequence or the 5' end of env so as to change the codon usage but not the amino acid sequence of the encoded proteins.

Secondly, the possibility of replication competent secondary  
30 viral vectors may be avoided by pseudotyping the genome of one

retrovirus with the envelope protein of another retrovirus or another enveloped virus so that regions of homology between the env and gag-pol components are avoided. In a particular embodiment the retroviral vector is constructed from the following three components. Transcription unit 1  
 5 contains a retroviral gag-pol gene under the control of a non-retroviral promoter and enhancer. Transcription unit 2 contains the env gene from the alternative enveloped virus, under the control of a non-retroviral promoter and enhancer. Transcription unit 3 comprises a defective retroviral genome under the control of a non-retroviral promoter and  
 10 enhancer. The defective retroviral genome contains a minimal packaging signal which does not contain sequences homologous to gag sequences in transcription unit 1.

Pseudotyping may involve for example a retroviral genome based on a lentivirus such as an HIV or equine infectious anaemia virus  
 15 (EIAV) and the envelope protein may for example be the amphotropic envelope protein designated 4070A. Alternatively, the retroviral genome may be based on MMLV and the envelope protein may be a protein from another virus which can be produced in non-toxic amounts within the  


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 primary target cell such as an Influenza haemagglutinin or vesicular  
 20 stomatitis virus G protein. In another alternative, the envelope protein may be a modified envelope protein such as a mutant or engineered envelope protein. Modifications may be made or selected to introduce targeting ability or to reduce toxicity or for another purpose.

Thirdly, the possibility of replication competent retroviruses  
 25 can be eliminated by using two transcription units constructed in a particular way. The first transcription unit contains a gag-pol coding region under the control of a promoter-enhancer active in the primary target cell such as a hCMV promoter-enhancer or a tissue restricted promoter-enhancer. The second transcription unit encodes a retroviral genome RNA  
 30 capable of being packaged into a retroviral particle. The second

transcription unit contains retroviral sequences necessary for packaging, integration and reverse transcription and also contains sequences coding for an env protein of an enveloped virus and the coding sequence of one or more therapeutic genes.

5                   A particularly preferred arrangement in this third example is one in which the transcription of the env and therapeutic gene coding sequences, or alternatively intron splicing, is devised such that the env protein is preferentially produced in the primary target cell while the therapeutic protein or proteins is or are preferentially produced in the  
10 secondary target cell. A suitable intron splicing arrangement is described in example 3 and illustrated in figure 3. A splice donor site is positioned downstream of the splice acceptor site in the retroviral genome sequence delivered by the primary vector to the primary target cell. Splicing will therefore be absent or infrequent in the primary target cell so envelope  
15 protein will preferentially be expressed. However, once the vector genome has gone through the process of reverse transcription and integration into the secondary target cell, the splice donor site will have been copied during that process so that it is located also in the 5' LTR, upstream of the splice acceptor sequence. Splicing occurs to splice out the envelope sequence  
20 and transcripts of the therapeutic gene are produced.

                  In a second preferred arrangement of this third example, the expression of a therapeutic gene is restricted to the secondary target cell and prevented from being expressed in the primary target cell as follows (illustrated in Figure 4). A promoter-enhancer and a first fragment of the  
25 therapeutic gene containing the 5' end of the coding sequence and a natural or artificially introduced splice donor site are placed in the 3'LTR of the retroviral genome construct upstream of the R-region. A second fragment of the therapeutic gene which contains all the sequences required to complete the coding region is placed downstream of a natural  
30 or artificially constructed splice acceptor site downstream from the

packaging signal in the retroviral genome construct. On reverse transcription and integration of the retroviral genome in the secondary target cell, the promoter 5' fragment of the therapeutic gene and splice donor come to reside upstream of the splice acceptor and 3' end of the therapeutic gene. Transcription from the promoter and splicing then permit translation of the therapeutic protein in the secondary target cell.

In a preferred embodiment the hybrid viral vector system according to the invention comprises single or multiple adenoviral primary vectors which encodes or encode a retroviral secondary vector.

Adenoviral vectors for use in the invention may be derived from a human adenovirus or an adenovirus which does not normally infect humans. Preferably the vectors are derived from Adenovirus Type 2 or adenovirus Type 5 (Ad2 or Ad5) or a mouse adenovirus or an avian adenovirus such as CELO virus (Cotton et al 1993 J. Virol. 67:3777-3785). The vectors may be replication competent adenoviral vectors but are more preferably defective adenoviral vectors. Adenoviral vectors may be rendered defective by deletion of one or more components necessary for replication of the virus. Typically, each adenoviral vector contains at least a deletion in the E1 region. For production of infectious adenoviral vector particles, this deletion may be complemented by passage of the virus in a human embryo fibroblast cell line such as human 293 cell line, containing an integrated copy of the left portion of Ad5, including the E1 gene. The capacity for insertion of heterologous DNA into such vectors can be up to approximately 7kb. Thus such vectors are useful for construction of a system according to the invention comprising three separate recombinant vectors each containing one of the essential transcription units for construction of the retroviral secondary vector.

Alternative adenoviral vectors are known in the art which contain further deletions in other adenoviral genes and these vectors are also suitable for use in the invention. Several of these second generation

adenoviral vectors show reduced immunogenicity (eg E1 + E2 deletions Gorziglia et al 1996 J. Virol. 70: 4173-4178; E1 + E4 deletions Yeh et al 1996 J. Virol. 70: 559-565). Extended deletions serve to provide additional cloning capacity for the introduction of multiple genes in the vector. For  
5 example a 25 kb deletion has been described (Lieber et al. 1996 J. Virol. 70: 8944-8960) and a cloning vector deleted of all viral genes has been reported (Fisher et al 1996 Virology 217: 11-22.) which will permit the introduction of more than 35kb of heterologous DNA. Such vectors may be used to generate an adenoviral primary vector according to the invention  
10 encoding two or three transcription units for construction of the retroviral secondary vector.

Embodiments of the invention described solve one of the major problems associated with adenoviral and other viral vectors, namely that gene expression from such vectors is transient. The retroviral particles  
15 generated from the primary target cells can infect secondary target cells and gene expression in the secondary target cells is stably maintained because of the integration of the retroviral vector genome into the host cell genome. The secondary target cells do not express significant amounts of  
viral protein antigens and so are less immunogenic than the cells  
20 transduced with adenoviral vector.

The use of a retroviral vector as the secondary vector is also advantageous because it allows a degree of cellular discrimination, for instance by permitting the targeting of rapidly dividing cells. Furthermore, retroviral integration permits the stable expression of therapeutic genes in  
25 the target tissue, including stable expression in proliferating target cells.

Preferably, the primary viral vector preferentially infects a certain cell type or cell types. More preferably, the primary vector is a targeted vector, that is it has a tissue tropism which is altered compared to the native virus, so that the vector is targeted to particular cells. The term  
30 "targeted vector" is not necessarily linked to the term "target cell". "Target

cell" simply refers to a cell which a vector, whether native or targeted, is capable of infecting or transducing.

The preferred, adenoviral primary vector according to the invention is also preferably a targeted vector, in which the tissue tropism of the vector is altered from that of a wild-type adenovirus. Adenoviral vectors can be modified to produce targeted adenoviral vectors for example as described in Krasnykh et al. 1996 J. Virol 70: 6839-6846; Wickham et al 1996 J. Virol 70: 6831-6838; Stevenson et al. 1997 J. Virol. 71: 4782-4790; Wickham et al. 1995 Gene Therapy 2: 750-756; Douglas et al. 1997 Neuromuscul. Disord. 7:284-298; Wickham et al. 1996 Nature Biotechnology 14: 1570-1573.

Primary target cells for the vector system according to the invention include but are not limited to haematopoietic cells (including monocytes, macrophages, lymphocytes, granulocytes or progenitor cells of any of these); endothelial cells; tumour cells; stromal cells; astrocytes or glial cells; muscle cells; and epithelial cells.

Thus, a primary target cell according to the invention, capable of producing the second viral vector, may be of any of the above cell types.

In a preferred embodiment, the primary target cell according to the invention is a monocyte or macrophage infected by a defective adenoviral vector containing a first transcription unit for a retroviral gag-pol and a second transcription unit capable of producing a packageable defective retroviral genome. In this case at least the second transcription unit is preferably under the control of a promoter-enhancer which is preferentially active in a diseased location within the body such as an ischaemic site or the micro-environment of a solid tumour. In a particularly preferred embodiment of this aspect of the invention, the second transcription unit is constructed such that on insertion of the genome into the secondary target cell, an intron is generated which serves to reduce expression of the viral env gene and permit efficient expression of a therapeutic gene.

The secondary viral vectors may also be targeted vectors. For retroviral vectors, this may be achieved by modifying the envelope protein. The envelope protein of the retroviral secondary vector needs to be a non-toxic envelope or an envelope which may be produced in non-toxic amounts within the primary target cell, such as for example a MMLV amphotropic envelope or a modified amphotropic envelope. The safety feature in such a case is preferably the deletion of regions or sequence homology between retroviral components.

The secondary target cell population may be the same as the primary target cell population. For example delivery of a primary vector of the invention to tumour cells leads to replication and generation of further vector particles which can transduce further tumour cells. Alternatively, the secondary target cell population may be different from the primary target cell population. In this case the primary target cells serve as an endogenous factory within the body of the treated individual and produce additional vector particles which can infect the secondary target cell population. For example, the primary target cell population may be

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haematopoietic cells transduced by the primary vector *in vivo* or *ex vivo*.

The primary target cells are then delivered to or migrate to a site within the body such as a tumour and produce the secondary vector particles, which are capable of transducing for example tumour cells within a solid tumour.

The invention permits the localised production of high titres of defective retroviral vector particles *in vivo* at or near the site at which action of a therapeutic protein or proteins is required with consequent efficient transduction of secondary target cells. This is more efficient than using either a defective adenoviral vector or a defective retroviral vector alone.

The invention also permits the production of retroviral vectors such as MMLV-based vectors in non-dividing and slowly-dividing cells *in vivo*. It had previously been possible to produce MMLV-based retroviral vectors only in rapidly dividing cells such as tissue culture-adapted cells

proliferating *in vitro* or rapidly dividing tumour cells *in vivo*. Extending the range of cell types capable of producing retroviral vectors is advantageous for delivery of genes to the cells of solid tumours, many of which are dividing slowly, and for the use of non-dividing cells such as endothelial  
 5 cells and cells of various haematopoietic lineages as endogenous factories for the production of therapeutic protein products.

The delivery of one or more therapeutic genes by a vector system according to the invention may be used alone or in combination with other treatments or components of the treatment. Diseases which  
 10 may be treated include, but are not limited to: cancer, neurological diseases, inherited diseases, heart disease, stroke, arthritis, viral infections and diseases of the immune system. Suitable therapeutic genes include those coding for tumour suppressor proteins, enzymes, pro-drug activating enzymes, immunomodulatory molecules, antibodies, engineered  
 15 immunoglobulin-like molecules, fusion proteins, hormones, membrane proteins, vasoactive proteins or peptides, cytokines, chemokines, anti-viral proteins, antisense RNA and ribozymes.

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~~In a preferred embodiment of a method of treatment~~

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according to the invention, a gene encoding a pro-drug activating enzyme  
 20 is delivered to a tumour using the vector system of the invention and the patient is subsequently treated with an appropriate pro-drug. Examples of pro-drugs include etoposide phosphate (used with alkaline phosphatase Senter et al., 1988 Proc. Natl. Acad. Sci. 85: 4842-4846); 5-fluorocytosine (with Cytosine deaminase Mullen et al. 1994 Cancer Res. 54: 1503-1506);  
 25 Doxorubicin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase (Kerr et al. 1990 Cancer Immunol. Immunother. 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with Carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with  $\beta$ -lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli et  
 30 al. 1988 Proc. Natl. Acad. Sci. 85: 7572-7576) mustard pro-drugs with

nitroreductase (Friedlos et al. 1997J Med Chem 40: 1270-1275) and Cyclophosphamide or Ifosfamide (with a cytochrome P450 Chen et al. 1996 Cancer Res 56: 1331-1340).

Further provided according to the invention are methods of  
5 controlling production of a therapeutic gene such that the therapeutic gene is preferentially expressed in the secondary target cell population and is poorly expressed or not expressed at a biologically significant level in the primary target cell.

In accordance with the invention, standard molecular biology  
10 techniques may be used which are within the level of skill in the art. Such techniques are fully described in the literature. See for example; Sambrook et al. (1989) Molecular Cloning; a laboratory manual; Hames and Glover (1985 - 1997) DNA Cloning: a practical approach, Volumes I-IV (second edition). Methods for the engineering of immunoglobulin genes  
15 in particular are given in McCafferty et al (1996) Antibody engineering: a practical approach.

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## EXAMPLES

### 20 **Example 1 – Construction of an MMLV amphotropic env gene with minimal homology to the Pol gene and a gag-pol transcription cassette**

In the Moloney murine leukaemia virus (MMLV), the first  
25 approximately 60 bps of the env coding sequence overlap with sequences at the 3' – end of the Pol gene. The region of homology between these two genes was removed to prevent the possibility of recombination between them in cells expressing both genes.

The DNA sequence of the first 60 bps of the coding  
30 sequence of env was changed while retaining the amino acid sequence of

the encoded protein as follows. A synthetic oligonucleotide was constructed to alter the codon usage of the 5'-end of env. (See figure 1.) and inserted into the remainder of env as follows.

The starting plasmid for re-construction of the 5' end of the  
 5 4070A gene was the pCI plasmid (Promega) into which had previously  
 been cloned the Xba1-Xba1 fragment containing the 4070A gene from  
 pHIT456 (Soneoka et al 1995 Nucl. Acids Res. 23: 628-633) to form pCI-  
 4070A. A PCR reaction was performed with primers A and B (Figure 1) on  
 pCI-4070A to produce a 600 base pair product. This product was then  
 10 cloned between the Nhe1 and Xho1 sites of pCI-4070A. The resulting  
 construct was sequenced across the Nhe1/Xho1 region. Although the  
 amino acid sequence of the resulting gene is the same as the original  
 4070A, the region of homology with the pol gene is removed.

15 The complete sequence of the modified env gene m4070A is  
 given in Figure 2. This sequence is inserted into the expression vector pCI  
 (Promega) by standard techniques.

---

20 The CMV gag-pol transcription unit is obtained from pHIT60  
 (Soneoka et al. 1995 Nucl. Acids Res. 23: 628-633).

### **Example 2 - Deletion of gag sequences from the retroviral packaging signal.**

25 A DNA fragment containing the LTR and minimal functional  
 packaging signal is obtained from the retroviral vector MFG (Bandara et al  
 1993 Proc. Natl. Acad. Sci 90: 10764-10768) or MMLV proviral DNA by  
 PCR reaction using the following oligonucleotide primers:

30 HindIIIIR: GCATTAAAGCTTTGCTCT

L523: GCCTCGAGCAAAAATTCAGACGGA

This PCR fragment contains MMLV nucleotides +1 to +523  
 5 and thus does not contain gag coding sequences which start at +621  
 (numbering based on the nucleotide sequence of MMLV Shinnick et al  
 1981 Nature 293: 543-548).

The PCR fragment can be used to construct a retroviral  
 10 genome vector by digestion using HindIII and XhoI restriction enzymes  
 and sub-cloning using standard techniques. Such vectors contain no  
 homology with gag coding sequences.

### Example 3 – Construction of defective retroviral genome

15

The transcription unit capable of producing a defective  
 retroviral genome is shown in Figure 3. It contains the following elements:  
 a hypoxia regulated promoter enhancer comprising 3 copies of the PGK –  
 gene HRE and a SV40 promoter deleted of the 72bp-repeat enhancer from  
 pGL3 (Promega); a MMLV sequence containing R, U5 and the packaging  
 20 signal; the coding sequence of m4070A (Example 1); a splice acceptor; a  
 cloning site for insertion of a coding sequence for a therapeutic protein; the  
 polypyrimidine tract from MMLV; a second copy of the HRE-containing  
 promoter-enhancer; a splice donor site; and a second copy of R, U5.

25

On reverse transcription and integration of the vector into the  
 secondary target cell, the splice donor is introduced upstream of the env  
 gene causing it to be removed from mRNA by splicing and thereby  
 permitting efficient expression of the therapeutic gene only in the  
 secondary target cell. (See Figure 3).

30

#### Example 4 - Construction of a conditional expression vector for Cytochrome P450

Figure 4 shows the structure of retroviral expression vector  
 5 cDNA coding sequences from the cytochrome P450 gene in two halves  
 such that only upon transduction is the correct splicing achieved to allow  
 P450 expression. This therefore restricts expression to transduced cells.

1) The starting plasmid for the construction of this vector is  
 10 pLNSX (Miller and Rosman 1989 BioTechniques 7: 980-990). The natural  
 splice donor (...agGTaag...) contained within the packaging signal of  
 pLNSX (position 781/782) is mutated by PCR mutagenesis using the  
 ALTERED SITES II mutagenesis kit (Promega) and a synthetic  
 oligonucleotide of the sequence:

15 5'-caaccaccgggagGCaagctggccagcaactta-3'

---

2) A CMV promoter from the pCI expression vector (Promega) is  
 isolated by PCR using the following two oligonucleotides:

20

Primer 1: 5'-atcggctagcagatcttcaatattggccattagccatat-3'

Primer 2: 5'-atcgagatctgcggccgcttacctgcccagtgccctcacgaccaa-3'

This produces a fragment containing the CMV promoter with  
 25 a 5'Nhe1 site (Primer 1) and a 3' Not1 and Xba1 site (Primer 2). It is cut  
 with Nhe1 and Xba1 and cloned into pLNSX from which an Nhe1-Nhe1  
 fragment has been removed.

3) The 5' end of a cytochrome P450 cDNA coding sequence is  
 30 isolated by RT-PCR from human liver RNA (Clontech) with the following

primers:

Primer 3: 5'-atcggcgggccgcccaccatggaactcagcgctcctccttctccttgccaccctagg-3'

Primer 4: 5'-atcggcgggccgcacttacCtgtgtgccccaggaaagtattcaagaagccag-3'

5

This amplifies the 5' end of the p450 from the ATG to residue 693 (numbering from the translation initiation site Yamano et al 1989 Biochem. 28:7340-7348). Contained on the 5' end of the fragment (derived from Primer 3) is also a Not1 site and an optimised "Kozak" translation initiation signal. Contained on the 3' end of the sequence (derived from primer 4) is another Not1 site and a consensus splice donor sequence (also found in pCI and originally derived from the human beta globin gene) with the GT splice donor pair located flush against residue 704 of P450 (the complementary residue is shown in uppercase in Primer 4). This fragment is digested with Not1 and cloned into the Not1 digested plasmid generated in step 2.

4) The Nhe1-Nhe1 fragment removed during the cloning of step 2 is then re-introduced into the plasmid of step 3. This creates a retroviral vector as described in Figure 3 but missing the 3' end P450.

5) The 3' of the P450 coding sequence is isolated by RT-PCR amplification from human liver RNA (Clontech) using the following primers:

25 Primer 5:

actgtgatcataggcacctattggtcttactgacatccactttctctccacagGcaagttacaaaacctgc  
aggaaatcaatgcttacatt-3'

Primer 6:

30 actgatcgattccctcagccccctcagcggggcaggaagc-3'

This generates the PCR amplified 3' end of P450 from residue 705 (in uppercase primer 5) and extends past the translation termination codon. Contained within the 5' end of this product and generated by primer 5 is a Bcl1 restriction site and a consensus splice  
5 acceptor and branch point (also found in pCI and originally from an immunoglobulin gene) upstream of residue 705. Contained at the 3' end of this product downstream of the stop codon and generated by primer 6 is a Cla1 site. This PCR product is then digested with Bcl1 and Cla1 and cloned into the vector of step 3 with the Bcl1-Cla1 fragment removed to  
10 generate the retroviral vector as shown in Figure 4.

---

FIGURE 1

5

SEQUENCE COMPARISON OF MUTANT ENV (m4070A) WITH WILD TYPE MMLV  
SEQUENCE FROM THE 3'-END OF THE POL GENE

10

3' POL        ATG GCG CGT TCA ACG CTC TCA AAA CCC CTT AAA AAT AAG  
GTT

5' m4070A    ATG GCC AGA AGC ACC CTG AGC AAG CCA CCC CAG GAC AAA  
ATC

15

                 \*\*\* \*\*       \*           \*\*    \*\*           \*\*    \*\*    \*       \*       \*

\*\*    \*

3' POL        AAC CCG CGA GGC CCC CTA ATC CCC

5' m4070A    AAT CCC TGG AAA CCT CTG ATC GTC

20

                 \*\*           \*\*           \*\*    \*\*    \*\*\*       \*

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Figure 2

1	CAGCTAGCTC	TAGACCACCA	TGGCCAGAAG	CACCCTGAGC	AAGCCACCCC
51	AGGACAAAAT	CAATCCCTGG	AAACCTCTGA	TCGTCATGGG	AGTCCTGTTA
101	GGAGTAGGGA	TGGCAGAGAG	CCCCCATCAG	GTCTTTAATG	TAACCTGGAG
151	AGTCACCAAC	CTGATGACTG	GGCGTACCGC	CAATGCCACC	TCCCTCCTGG
201	GAACTGTACA	AGATGCCTTC	CCAAAATTAT	ATTTTGATCT	ATGTGATCTG
251	GTCGGAGAGG	AGTGGGACCC	TTCAGACCAG	GAACCGTATG	TCCGGGTATGG
301	CTGCAAGTAC	CCCGCAGGGA	GACAGCGGAC	CCGGACTTTT	GACTTTTACG
351	TGTGCCCTGG	GCATACCGTA	AAGTCGGGGT	GTGGGGGACC	AGGAGAGGGC
401	TACTGTGGTA	AATGGGGGTG	TGAAACCACC	GGACAGGCTT	ACTGGAAGCC
451	CACATCATCG	TGGGACCTAA	TCTCCCTTAA	GCGCGGTAAC	ACCCCTGGG
501	ACACGGGATG	CTCTAAAGTT	GCCTGTGGCC	CCTGCTACGA	CCTCTCCAAA
551	GTATCCAATT	CCTTCCAAGG	GGCTACTCGA	GGGGGCAGAT	GCAACCTCT
601	AGTCCTAGAA	TTCAGTATG	CAGGAAAAAA	GGCTAACTGG	GACGGGCCCCA
651	AATCGTGGGG	ACTGAGACTG	TACCGGACAG	GAACAGATCC	TATTACCATG
701	TTCTCCCTGA	CCCGGCAGGT	CCTTAATGTG	GGACCCCGAG	TCCCCATAGG
751	GCCCAACCCA	GTATTACCCG	ACCAAAGACT	CCCTTCCTCA	CCAATAGAGA
801	TTGTACCGGC	TCCACAGCCA	CCTAGCCCCC	TCAATACCAG	TTACCCCCCT
851	TCCACTACCA	GTACACCCTC	AACCTCCCCT	ACAAGTCCAA	GTGTCCCACA
901	GCCACCCCCA	GGAAGTGGAG	ATAGACTACT	AGCTCTAGTC	AAAGGAGCCT
951	ATCAGGCGCT	TAACCTCACC	AATCCCGACA	AGACCCAAGA	ATGTTGGCTG
1001	TGCTTAGTGT	CGGGACCTCC	TTATTACGAA	GGAGTAGCGG	TCGTGGGCAC
1051	TTATACCAAT	CATTCCACCG	CTCCGGCCAA	CTGTACGGCC	ACTTCCCAAC
1101	ATAAGCTTAC	CCTATCTGAA	GTGACAGGAC	AGGGCCTATG	CATGGGGGCA
1151	GTACCTAAAA	CTCACCAGGC	CTTATGTAAC	ACCACCCAAA	GCGCCGGCTC
1201	AGGATCCTAC	TACCTTGCAG	CACCCGCCGG	AACAATGTGG	GCTTGCAGCA
1251	CTGGATTGAC	TCCCTGCTTG	TCCACCACGG	TGCTCAATCT	AACCACAGAT
1301	TATTGTGTAT	TAGTTGAACT	CTGGCCCAGA	GTAATTTACC	ACTCCCCCGA
1351	TTATATGTAT	GGTCAGCTTG	AACAGCGTAC	CAAATATAAA	AGAGAGCCAG
1401	TATCATTGAC	CCTGGCCCTT	CTACTAGGAG	GATTAACCAT	GGGAGGGATT
1451	GCAGCTGGAA	TAGGGACGGG	GACCACTGCC	TTAATTAAAA	CCCAGCAGTT
1501	TGAGCAGCTT	CATGCCGCTA	TCCAGACAGA	CCTCAACGAA	GTGGAAGAAGT
1551	CAATTACCAA	CCTAGAAAAG	TCACTGACCT	CGTTGTCTGA	AGTAGTCCTA
1601	CAGAACCGCA	GAGGCCTAGA	TTTGCTATTC	CTAAAGGAGG	GAGGTCTCTG
1651	CGCAGCCCTA	AAAGAAGAAT	GTTGTTTTTA	TGCAGACCAC	ACGGGGGCTAG
1701	TGAGAGACAG	CATGGCCAAA	TTAAGAGAAA	GGCTTAATCA	GAGACAAAAA
1751	CTATTTGAGA	CAGGCCAAGG	ATGGTTCGAA	GGGCTGTTTA	ATAGATCCCC
1801	CTGGTTTACC	ACCTTAATCT	CCACCATCAT	GGGACCTCTA	ATAGTACTCT
1851	TACTGATCTT	ACTCTTTGGA	CCTTGCATTG	TCAATCGATT	GGTCCAATTT
1901	GTTAAAGACA	GGATCTCAGT	GGTCCAGGCT	CTGGTTTTGA	CTCAGCAATA
1951	TCACCAGCTA	AAACCCATAG	AGTACGAGCC	ATGA	

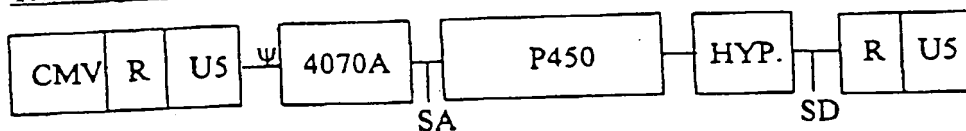
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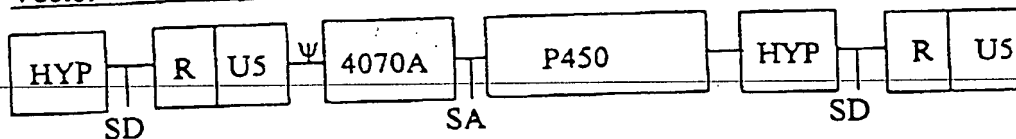
Figure 3

# RESTRICTED GENE EXPRESSION CONSTRUCT

Transcription unit in primary target cell



Vector DNA integrated into secondary target cell

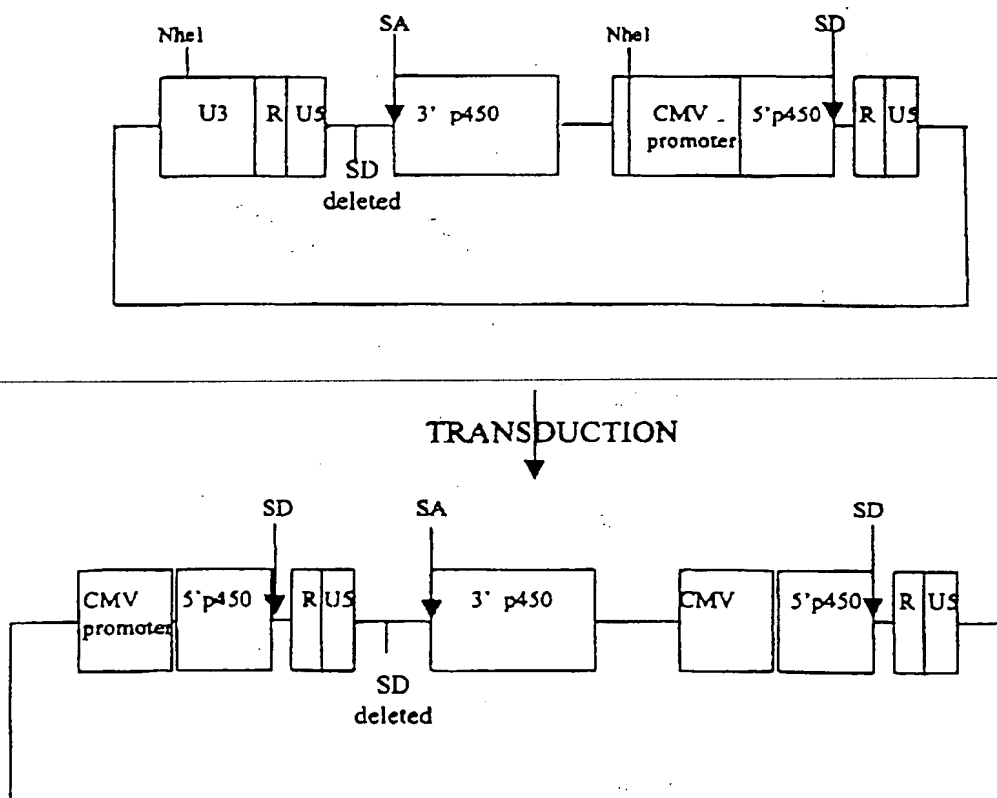


CMV	= CMV Promoter
HYP	= Hypoxia responsive promoter
Ψ	= MLV packaging signal
4070A	= MLV amphotrophic Env gene
p450	= cDNA encoding cytochrome P450
SD	= Splice donor
SA	= Splice acceptor

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Figure 4: Use of an intron to restrict p450 expression to transduced cell



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